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Expression pattern in retinal photoreceptors of POMGnT1, a protein involved in muscle-eye-brain disease

Mary Luz Uribe,[1,2] Carmen Haro,[1] María Paz Ventero,[1] Laura Campello,[1] Jesús Cruces,[2,3] José Martín-Nieto[1,2,4]

[1]Departamento de Fisiología, Genética y Microbiología, Facultad de Ciencias, Universidad de Alicante, Alicante, Spain; [2]Instituto de Investigación Sanitaria del Hospital Universitario La Paz (IdiPAZ), Madrid, Spain; [3]Departamento de Bioquímica, Instituto de Investigaciones Biomédicas 'Alberto Sols' UAM-CSIC, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain; [4]Instituto Multidisciplinar para el Estudio del Medio (IMEM) 'Ramón Margalef', Universidad de Alicante, Alicante, Spain

Purpose: The *POMGNT1* gene, encoding protein O-linked-mannose β-1,2-N-acetylglucosaminyltransferase 1, is associated with muscle-eye-brain disease (MEB) and other dystroglycanopathies. This gene's lack of function or expression causes hypoglycosylation of α-dystroglycan (α-DG) in the muscle and the central nervous system, including the brain and the retina. The ocular symptoms of patients with MEB include retinal degeneration and detachment, glaucoma, and abnormal electroretinogram. Nevertheless, the POMGnT1 expression pattern in the healthy mammalian retina has not yet been investigated. In this work, we address the expression of the *POMGNT1* gene in the healthy retina of a variety of mammals and characterize the distribution pattern of this gene in the adult mouse retina and the 661W photoreceptor cell line.

Methods: Using reverse transcription (RT)–PCR and immunoblotting, we studied *POMGNT1* expression at the mRNA and protein levels in various mammalian species, from rodents to humans. Immunofluorescence confocal microscopy analyses were performed to characterize the distribution profile of its protein product in mouse retinal sections and in 661W cultured cells. The intranuclear distribution of POMT1 and POMT2, the two enzymes preceding POMGnT1 in the α-DG O-mannosyl glycosylation pathway, was also analyzed.

Results: *POMGNT1* mRNA and its encoded protein were expressed in the neural retina of all mammals studied. POMGnT1 was located in the cytoplasmic fraction in the mouse retina and concentrated in the myoid portion of the photoreceptor inner segments, where the protein colocalized with GM130, a Golgi complex marker. The presence of POMGnT1 in the Golgi complex was also evident in 661W cells. However, and in contrast to retinal tissue, POMGnT1 additionally accumulated in the nucleus of the 661W photoreceptors. Colocalization was found within this organelle between POMGnT1 and POMT1/2, the latter associated with euchromatic regions of the nucleus.

Conclusions: Our results indicate that POMGnT1 participates not only in the synthesis of O-mannosyl glycans added to α-DG in the Golgi complex but also in the glycosylation of other yet-to-be-identified proteins in the nucleus of mouse photoreceptors.

Proper O-mannosyl glycosylation is required for central nervous system (CNS) development and function, and it is known that O-mannose-initiated glycans comprise a large portion of the total O-glycans in the mammalian brain [1]. Mutations in genes involved in O-mannosylation give rise to congenital muscular dystrophies with associated CNS abnormalities [2-5]. A deficient glycosylation of dystroglycan (DG), which thus far is the best characterized O-mannosylated protein, accounts for the muscular and neurologic phenotypes associated with dystroglycanopathies [2,3,6,7]. This group of recessively inherited disorders is clinically characterized by various combinations of severe muscular dystrophy, mental retardation, and ocular abnormalities [2,3,5]. Thus far, mutations in 18 genes have been identified in patients with dystroglycanopathies, most of which code for glycosyltransferases, DG itself, or proteins of unknown function in DG glycosylation [8,9].

DG is a glycoprotein that constitutes the central component of the dystrophin-glycoprotein complex. This transmembrane multimeric complex is made up of dystrophin, sarcoglycan, sarcospan, and syntrophin, among other proteins, being responsible for the linkage of the cytoskeleton of muscle and nerve cells to the extracellular matrix [4,9,10]. These proteins play an important role in maintaining muscle integrity and are involved in proper CNS development, structure,

Correspondence to: José Martín-Nieto, Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Campus Universitario San Vicente, P.O. Box 99, E-03080 Alicante, Spain; Phone: +34-96-590-9766; FAX: +34-96-590-9569; email: jmnieto@ua.es

Ms. Ventero is now at Unidad de Investigación, Hospital General Universitario de Elche, Universidad Miguel Hernández, E-03202 Elche, Alicante, Spain

and function, myelination and architecture of peripheral nerves, epithelial morphogenesis, cell adhesion, and signal transduction, among other functions [10,11]. DG is translated from the *DAG1* gene (Gene ID 1605, OMIM 128239) as a precursor polypeptide, which through posttranslational, proteolytic cleavage is excised into two subunits, α-DG and β-DG. The first is extracellularly located and heavily glycosylated, and the second is a transmembrane polypeptide. α-DG glycosylation is essential for its proper functioning [5,9,10], as the attached O-mannosyl glycans confer α-DG the ability to bind to its protein ligands in the extracellular matrix of muscle and CNS tissues and to synaptic molecules, including laminin, perlecan, agrin, neurexin, pikachurin, and slit [9,11,12]. Among these molecules, pikachurin is exclusively expressed in the retina and mediates the proper synaptic connection between retinal photoreceptors and bipolar cells [13].

Mutations in *POMGNT1*, the first described dystroglycanopathy-associated gene [14], have been mainly associated with the development of muscle-eye-brain disease (MEB) [15-17], a severe neuromuscular dystrophy, although they have also been described in patients with Walker-Warburg syndrome (WWS) [18], both collected in the OMIM database under entry MDDGA3 (OMIM 253280), as well as with moderate congenital muscular dystrophy with mental retardation, type B3 (MDDGB3, OMIM 613151) [15,16], and with the milder limb-girdle muscular dystrophy (LMGD) with no CNS affectation, type 2O (MDDGC3, OMIM 613157) [19]. This gene encodes the enzyme dubbed protein O-linked-mannose β-1,2-N-acetylglucosaminyltransferase 1 (POMGnT1), contains 22 exons, and is located in chromosome region 1p34.1 (OMIM 606822). Transcription of the *POMGNT1* gene gives rise to a 2.7 kb mRNA in different tissues, with higher expression levels in the skeletal muscle, heart, and kidney and lower levels in the brain [14]. POMGnT1 (EC 2.4.1.101) is a protein belonging to the GT13 family of glycosyltransferases according to the Carbohydrate-Active enZYmes (CAZy) database [20]. In humans, the main isoform of POMGnT1 contains 660 amino acids whose sequence yields a calculated molecular mass of 75,252 Da (UniProtKB Q8WZA1). We have recently described the case of a patient with an MDDGC3 variant in which the causative mutation was not found in the *POMGNT1* coding sequence but in its promoter region [21]. This mutation causes underexpression of this gene at the mRNA level, leading to hypoglycosylation of α-DG and the development of MEB symptoms.

In addition to POMGnT1, enzyme activity has been demonstrated for other glycosyltransferases involved in dystroglycanopathies, such as protein O-mannosyltransferases POMT1 and POMT2 (EC 2.4.1.109). These are thought to form a heterodimer active in the addition of mannose to serine or threonine residues of α-DG [22,23], which are numerous and located in its mucin-like domain [9,10]. POMGnT1 then functions to catalyze the second step in the synthesis of α-DG O-mannosyl glycans (cores M1 and M2) by covalently attaching, with a β1–2 linkage, one unit of N-acetylglucosamine to the previously added O-mannose residue [11,14,24]. Thus far, POMGnT1 has been localized to the Golgi complex when exogenously expressed in a few established cell lines [25,26], and the protein has been recently shown to be associated with a Golgi-resident protein known as Golgi phosphoprotein 3 (GOLPH3) [27].

To investigate the in vivo function of POMGnT1, MEB mouse models have been generated through *Pomgnt1* gene inactivation that exhibit multiple defects in the muscle, brain, and eye, comparable to those experienced by people affected by MEB [28-30]. To date, the distribution of POMGnT1 in the vertebrate retina has not been studied, and the protein's pattern of expression and intracellular localization have not been determined in any mammalian tissue. In this work, we analyzed the expression of *POMGNT1* mRNA and protein in the retina of various mammalian species. We also focused on characterizing the distribution pattern in the neural retina of POMGnT1 in healthy adult mice and in the established cell line of cone photoreceptors, 661W. Interestingly, in addition to being found in the Golgi complex, POMGnT1 was found to concentrate in the nucleus of 661W cells. This led us to jointly assess the possible intranuclear presence of its preceding enzymes in α-DG O-glycosylation, POMT1 and POMT2, in this photoreceptor cell line.

METHODS

Animals: The following mammalian species studied in this work were all adults: mouse (*Mus musculus*, C57BL/6J), rat (*Rattus norvegicus*, Sprague-Dawley), cow (*Bos taurus*), cynomolgus monkey (*Macaca fascicularis*), and human (*Homo sapiens*). All animal handling was performed in compliance with the rules set by the National Institutes of Health (USA), the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the European Directive 2010/63/EU. Rodents were held at the Universidad de Alicante animal-care facility and euthanized upon CO_2 inhalation followed by cervical dislocation. Their eyes were enucleated and stored in RNAlater solution (Ambion; Austin, TX). Bovine eyes were freshly obtained from the Alicante municipal slaughterhouse. Monkeys were kept as described [31] at the animal care facility of the Universidad de Murcia (Spain), and their eyes were kindly donated by Dr. M.T.

Herrero. All protocols and primate handling procedures used were approved by the university bioethics research committee. Young adult monkeys were anesthetized with ketamine (10 mg/kg, i.m.) and then administered a lethal injection of pentobarbital (50 mg/kg, i.p.), and the eyeballs were immediately enucleated following sacrifice. All animal eyes were kept at –80 °C until the retinas were dissected for RNA or protein isolation. For immunohistochemistry, mouse eyes were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4 (PB) for 1 h and then subjected to sucrose cryoprotection [32].

Cell culture: The 661W photoreceptor cell line was kindly provided by Dr. M. Al-Ubaidi (University of Oklahoma). It was cloned from a retinal tumor of a transgenic mouse line that expressed the artificial *HIT1* transgene (SV40 large T antigen under control of the human inter-photoreceptor retinol-binding protein (*IRBP*) gene (Gene ID 5949, OMIM 180290) promoter) [33]. These immortalized cells express cone molecular markers, such as opsins, transducin, and cone arrestin, and lack proteins specific to rods or other retinal cell types, constituting a light-sensitive, homogeneous cell line [34,35]. The 661W cells were authenticated with short tandem repeats (STR) genotyping (IDEXX BioResearch, Ludwigsburg, Germany), and the genetic profile was verified to be consistent with a mixed FVB × C57BL/6 mouse strain of origin, as expected, and to carry the *HIT1* transgene, which makes this cell line unique (Appendix 1) [33]. The cells were grown on Dulbecco's modified Eagle medium (DMEM) from HyClone (Logan, UT), supplemented with 10% fetal bovine serum from Gibco (Grand Island, NY), 2 mM L-glutamine, and 1% penicillin/streptomycin (HyClone), at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were expanded on 75 cm^2 flasks, and after five to ten passages under culture conditions, the cells were frozen at –80 °C until RNA or protein extraction was performed, or else they were seeded on 24-well culture plates and used for immunocytochemistry when they were nearly confluent.

Reverse transcription–PCR: The neural retina was dissected out, free from the retinal pigment epithelium (RPE), from eyes stored in RNAlater. Total RNA was extracted from the retinal tissue and 661W cells using the TRIzol reagent (Invitrogen; Carlsbad, CA) and then treated with DNase I using the TURBO DNA-free kit (Ambion). RNA from human retinas (29 donors, 20–60 years at death) was purchased from Clontech BD (Mountain View, CA). Reverse transcription into cDNA was performed using the RETROscript kit (Ambion), and subsequent PCR amplification was performed essentially as described [36,37]. Primers for genes *POMGNT1*, *POMT1*, and *POMT2* (Table 1) were designed using the Primer3 software [38] to bear a T_m of 60 °C and flank at least one intron of the corresponding gene. PCR reactions (40 µl) contained 20 ng of cDNA, forward and reverse primers at 0.4 µM each, the four deoxynucleotides (dNTPs) at 0.2 mM each, and 1 U of GoTaq DNA polymerase (Promega; Madison, WI). After an initial denaturation step at 94 °C for 2 min, amplification was performed for a total of 35 cycles each at 94 °C for 30 s, 60 °C for 20 s, and 72 °C for 30 s, and concluded with a final elongation step at 72 °C for 5 min. PCR products were run on 2% agarose gels with added SYBR Green I (Sigma-Aldrich; St. Louis, MO) and photographed under ultraviolet (UV) light.

Immunoblotting: Total proteins were extracted from the neural retinas and 661W cells as previously described [36]. Briefly, 20 µl of lysis buffer supplemented with protease inhibitors was added per 5 mg of tissue or 10^6 cells, and after incubation on ice for 15–20 min, the supernatant obtained upon centrifugation at 16,000 ×*g* at 4 °C for 10 min constituted the total protein fraction. To obtain the cytoplasmic and nuclear protein fractions, the NE-PER kit from Pierce (Rockford, IL) was used. Samples (20 mg of retina or 2×10^6 cells) were homogenized in 200 µl of CER I lysis buffer supplemented with protease inhibitors and kept on ice for 10 min. Then 11 µl of CER II lysis buffer was added, and after centrifugation as above for 5 min, the supernatant obtained constituted the cytoplasmic fraction. To the pellet, a volume of 100 µl of cold NER lysis buffer was added, and after incubation on ice for 40 min, the supernatant obtained after centrifugation as above for 10 min constituted the nuclear fraction.

Proteins (100 µg/lane) were resolved with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 5–20% polyacrylamide-gradient gels and, after electrotransfer to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK), stained with SYPRO Ruby Protein Blot Stain (Molecular Probes; Eugene, OR) to ensure even protein loading and transfer. Immunodetection was performed essentially as described [31,36,37]. The membranes were probed sequentially with primary antibodies (Table 2) at 4 °C overnight and with horseradish peroxidase-conjugated anti-immunoglobulin (anti-IgG) secondary antibodies at a 1:10,000 dilution for 1 h. Development was performed with enhanced chemiluminescence using the SuperSignal kit from Pierce, upon exposure of the X-ray films (GE Healthcare) to immunoblots. Absence of cross contamination between the cytoplasmic and nuclear fractions was assessed with immunoblotting using antibodies to proteins exclusively found in these compartments, namely, β-tubulin III and lamin A/C, respectively (Table 2).

Table 1. Primers used for RT–PCR in this work.

Gene	Species	Primer Sequences	Exon	PCR product size (bp)
POMGNT1	Hu, Mk, Rt, Mo	Fw: 5‘-ATCCTACCACTTTGGCATCG-3′	17	108
		Rv: 5‘-ACATTCCTGAGCTGGACACC-3′	18	
	Bv	Fw: 5‘-ATCCTACCACTTCGGCATCG-3′	17	108
		Rv: 5‘-ACATTCCTGAGCTGGACACC-3′	18	
POMT1	Hu, Mk	Fw: 5‘-AATCATGTCCAGTGCCTTCC-3′	9	165
		Rv: 5‘-ATCATGGGGTAGGTGTCCTG-3′	10	
	Bv	Fw: 5‘-AATCATGTCCAGTGCCTTCC-3′	9	165
		Rv: 5‘-ATCATAGGGTAGGTGCTCTGGT-3′	10	
	Rt, Mo	Fw: 5‘-AATCATGTCCAGTGCCTTCC-3′	9	165
		Rv: 5‘-ATCATGGGATAGGTGTTCTTGT-3′	10	
POMT2	Hu, Mk	Fw: 5‘-GGTTTGGTGGCTGAATCTGT-3′	18	222
		Rv: 5‘-ATGGCTGGGAAGTAGTGGTG-3′	19	
	Bv	Fw: 5‘-TCCTGTGCCTCATAGTGCTG-3′	7	238
		Rv: 5‘-TAGAAGTGCCTGTGCGAGTG-3′	9	
	Rt	Fw: 5‘-TCCTGTGCCTCATAGTGCTG-3′	7	176
		Rv: 5‘-AGAGCCATACGCCAAGTGTT-3′	8-9	
	Mo	Fw: 5‘-GTCTGGTGGCTGAATCTGGT-3′	18	221
		Rv: 5‘-ATGGCTGGGAAGTAGTGGTG-3′	19	

The sequences of forward (Fw) and reverse (Rv) primers used in this work are indicated. The exon to which each primer aligns is indicated in the penultimate column. The PCR product size is indicated in the last column for each primer pair. Hu: human, Mk: monkey, Bv: bovine, Rt: rat, Mo: mouse. For the 661W cell line the mouse primers were used.

Table 2. Primary antibodies used in this work.

Protein	Antibody	Company	Catalog no.	Working dilution		
				IB	IHC	ICC
POMGnT1	Mouse, 6C12	Sigma-Aldrich	WH0055624M7	1:250	1:100	1:250
POMT1	Rabbit, polyclonal	Sigma-Aldrich	SAB2101845	1:1000	–	–
POMT1	Rabbit, polyclonal	Eurogentec[a]	SP26[a]	–	–	1:50
POMT2	Rabbit, polyclonal	Sigma-Aldrich	HPA003663	1:250	–	–
POMT2	Goat, polyclonal	Santa Cruz[b]	sc-48919	–	–	1:50
β-tubulin III	Rabbit, polyclonal	Sigma-Aldrich	T3952	1:1,000	–	–
Lamin A/C	Mouse, 4C11	Sigma-Aldrich	SAB200236	1:10,000	–	–
GM130	Goat, polyclonal	Santa Cruz	sc-16271	–	1:100	–
GM130	Rabbit, EP892Y	Abcam[c]	ab52649	–	–	1:250
Cone arrestin	Rabbit, polyclonal	Millipore[d]	AB15282	–	1:500	–
H3K4me3	Rabbit, polyclonal	Abcam	ab8580	–	–	1:100
HP1α	Goat, polyclonal	Abcam	ab77256	–	–	1:250

The two first columns list the antigen and animal origin of the antibody. The clone code is specified for monoclonal antibodies. The two central columns indicate the commercial company and antibody catalog reference. The three last columns specify the dilution at which each antibody was used on immunoblotting (IB), immunocytochemistry (ICC) or immunohistochemistry (IHC). [a]Eurogentec (Liège, Belgium), [b]Santa Cruz Biotechnology (Santa Cruz, CA), [c]Abcam (Cambridge, MA), [d]Merck Millipore (Darmstad, Germany).

Immunohistochemistry: Cryostat vertical sections (14–16 µm thick) were obtained and processed for immunohistochemistry essentially as described [32,36,37]. Sections were subjected to single or double immunostaining with primary antibodies (Table 2) in phosphate buffer (PB) supplemented with 1% (v/v) Triton X-100 (PBX) at room temperature overnight. Thereafter, the samples were incubated in the presence of secondary antibodies to IgG developed in donkey and conjugated to Alexa Fluor 488 (green) or 546 (red; Molecular Probes), at a 1:100 dilution in PBX. 4',6-Diamidino-2-phenylindole (DAPI) was simultaneously added at 10 µg/ml, and incubation took place for 1 h at room temperature with gentle shaking in the dark in a humidified chamber. Control samples in which primary antibodies were omitted and the corresponding secondary antibodies were included were processed in parallel. Fluorescence was detected with a Leica TCS SP2 confocal laser scanning microscope (Leica Microsystems; Wetzlar, Germany). Colocalization was assessed with the JACoP plugin developed for the NIH ImageJ software. A Pearson's coefficient (PC) close to 1 was taken as indicative of complete colocalization and close to 0 of no colocalization [39]. Each PC value was calculated as the average ± standard deviation (SD) from five cryosections, each belonging to a separate experiment, in which the inner segment (IS) layer was cropped before analysis.

Immunocytochemistry: The 661W photoreceptors were seeded on 24-well culture plates at 75,000 cells per well and grown on a 5% CO_2 atmosphere at 37 °C for 24 h. Then the cells were fixed with 4% paraformaldehyde in Dulbecco's PBS (DPBS) for 10 min. Thereafter, they were permeabilized in situ with 0.2% Triton X-100 in DPBS for 10 min, and then non-specific binding was prevented by incubation with 1% bovine serum albumin (BSA) in DPBS (blocking solution) for 1 h. Subsequently, the cells were incubated with primary antibodies at the dilutions indicated in Table 2 in blocking solution in a humidified chamber at 4 °C overnight. The SP26 antibody to POMT1 was custom-made against amino acids 510-524 of the human protein [40]. Thereafter, they were incubated with secondary antibodies to IgG at a 1:100 dilution and 10 µg/ml DAPI at room temperature for 2 h. Controls, fluorescence detection with confocal microscopy, and colocalization analyses were performed as indicated above. Each PC value was calculated as the average ± SD of ten cells from three micrographs, each obtained in a separate experiment. The 661W nuclei or Golgi complexes, as indicated in each case, were cropped before analysis.

RESULTS

To assess whether the gene encoding POMGnT1 is expressed in the mammalian retina, eyes from different species were enucleated, and the neural retina was dissected out for total RNA and protein isolation. With RT–PCR using primers specific for each mammal, we found that the *POMGNT1* gene was transcribed in the retina of all species, from rodents (mouse and rat) and bovine to primates (monkey and human; Figure 1A). Expression of this gene was also detected in the mouse cell line of cone photoreceptors, 661W. Next, we assessed with immunoblotting whether the protein product, POMGnT1, was synthesized in the retina and found coherent results with mRNA expression. As shown in Figure 1B, the POMGnT1 enzyme was detected in the retina of all mammalian species analyzed, as well as in the 661W photoreceptors, migrating with an apparent molecular mass of 75 kDa as expected from its amino acid sequence.

As POMT1 and POMT2 are known to precede POMGnT1 in the α-DG O-mannosyl glycosylation pathway, by adding to serine/threonine residues the O-mannose unit serving as a substrate for POMGnT1 enzyme activity, we sought to investigate whether the genes encoding those two proteins were also expressed in the mammalian retina. As was the case for POMGnT1, we found that the *POMT1* and *POMT2* genes were expressed at the mRNA level in the neural retina of all mammals studied, as well as in the 661W photoreceptor cell line (Figure 1A). In keeping with these results, their encoded protein products, POMT1 and POMT2, were found to be present as well in the retinas of all mammals enlisted above and in the 661W cells (Figure 1B). Molecular masses for these proteins were, as expected, 82 and 84–85 kDa (depending on the species), respectively.

The intracellular localization of POMGnT1 in retinal cells is unknown. To investigate whether its distribution was cytoplasmic or nuclear or both, these protein fractions were extracted from the adult mouse retina and the 661W cells. As shown in Figure 1C, immunoblotting analysis revealed that POMGnT1 was distributed in the retina exclusively in the cytoplasm, whereas in the 661W photoreceptors this enzyme was found in the cytoplasmic and nuclear fractions. By using specific antibodies against β-tubulin III (an exclusively-cytoplasmic protein) and lamin A/C (an exclusively-nuclear protein), we ascertained that the procedure we followed did not give cross contamination between the two fractions (Figure 1C).

Next, we set out to determine the distribution profile of POMGnT1 in retinal tissue. With this purpose, we performed immunofluorescence confocal microscopy analysis on retinal sections from adult mice. As shown, POMGnT1 localization

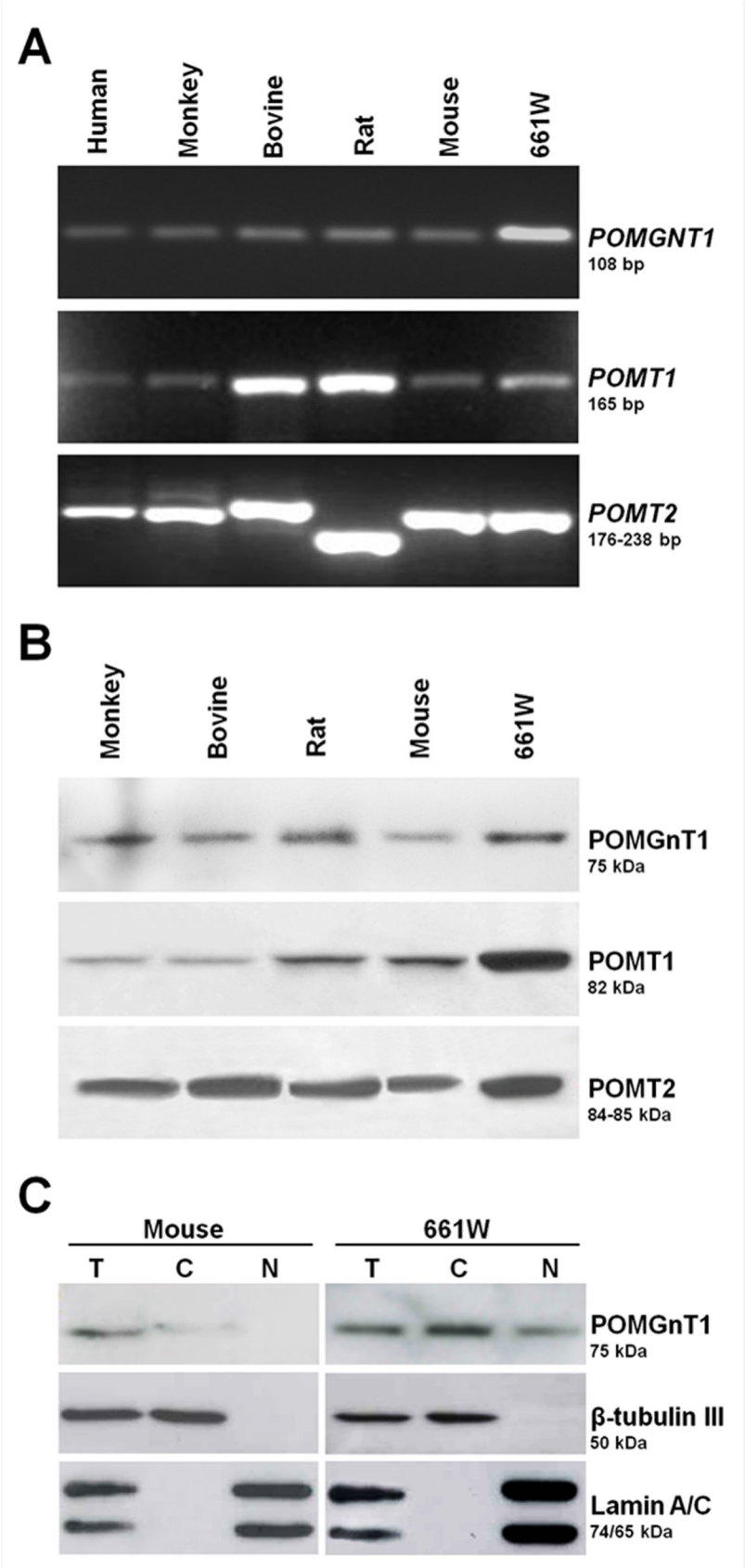

Figure 1. Expression of *POMGNT1*, *POMT1*, and *POMT2* at the mRNA and protein levels in the mammalian retina and in the 661W cell line of cone photoreceptors. **A**: Total RNA was extracted from the neural retina of the species indicated and the 661W cell line. Expression of mRNAs encoded by the three genes was assessed with reverse transcription (RT)–PCR using specific primers. **B**: Immunoblotting analysis of the expression of POMGnT1, POMT1, and POMT2 on total protein extracts from the neural retina of the species listed and the 661W cell line. **C**: Total (T), cytoplasmic (C), and nuclear (N) protein fractions from the mouse neural retina and 661W cells were obtained, and immunoblotting was performed using antibodies to POMGnT1, β-tubulin III (a control cytoplasmic protein), and lamin A/C (two control, related nuclear proteins). The molecular sizes of the DNA (**A**) or protein (**B**, **C**) bands obtained are indicated to the right of each panel.

was observed only in the photoreceptor IS layer (Figure 2A,E), where immunoreactivity concentrated in a punctate band immediately above the photoreceptor nuclei stained with DAPI in the outer nuclear layer (Figure 2B). The signal from the retinal vessels was non-specific, as it persisted when the primary antibody was omitted (Figure 2C). Immunostaining with an antibody specific to cone arrestin revealed that POMGnT1 was located in the myoid region (Figure 2D), the photoreceptor IS portion where the endoplasmic reticulum and the Golgi complex are contained. To discern between these organelles, double immunostaining was performed by using antibodies to POMGnT1 together with an antibody against GM130 (Figure 2F), a structural protein of the Golgi apparatus widely used as a marker for this organelle. Significant colocalization of the immunolabeling was found all along the IS layer (Figure 2G,H, yellow; PC = 0.69±0.04). Localization of the Golgi complex in the myoid of the cone photoreceptors is illustrated in Figure 2I.

POMGNT1 was expressed in the photoreceptor cell line 661W (Figure 1A,B) and the protein product distributed between the nucleus and the cytoplasm (Figure 1C). To further assess the intracellular localization of POMGNT1, cells were immunostained with anti-POMGnT1 antibodies and visualized with fluorescence microscopy. In keeping with immunoblotting data, it was also found that POMGnT1 was present in both cellular compartments (Figure 3A). A higher immunoreactivity density was found for POMGnT1 inside the nucleus, in the form of a punctate pattern, although without colocalization with DAPI-stained DNA in this compartment (Figure 3B,C,H; PC = 0.35±0.04). Immunolabeling in the cytoplasm and the nucleus was lost when the primary antibody was omitted (Figure 3D). In the cytoplasm, this enzyme

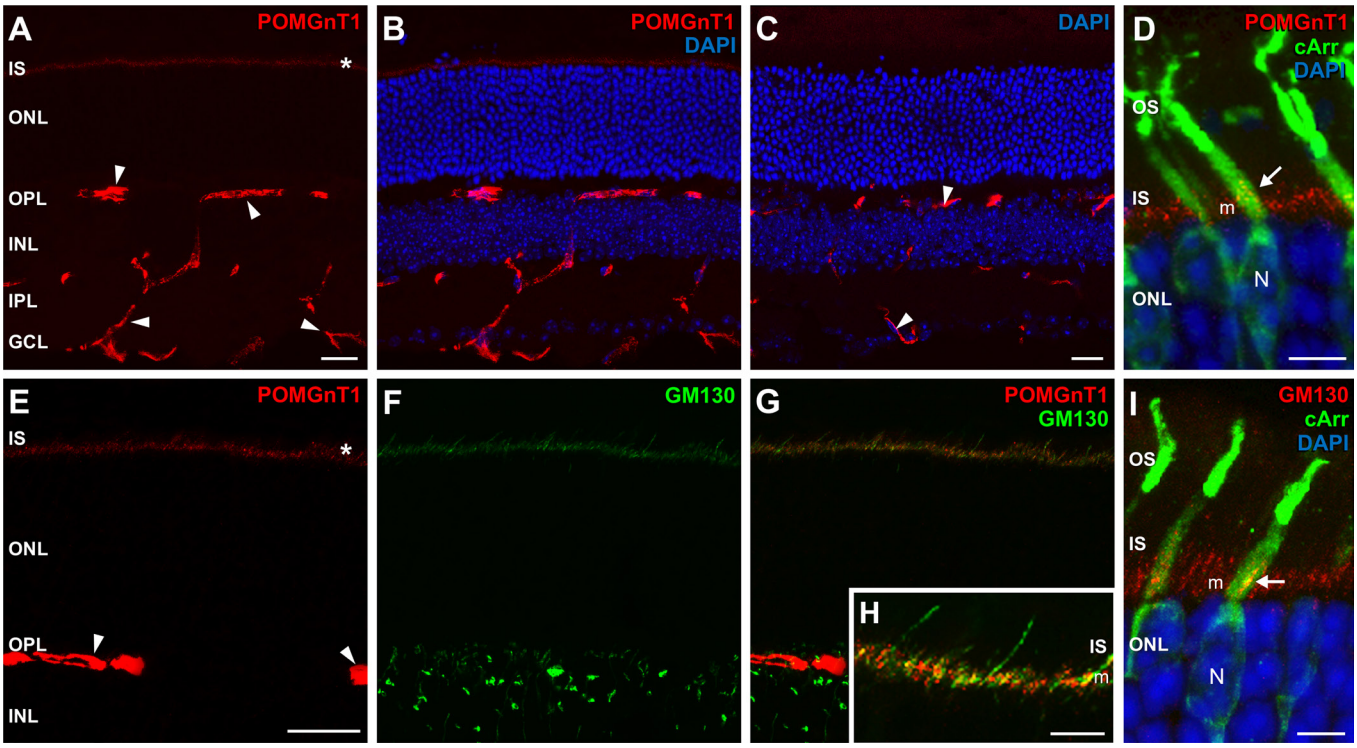

Figure 2. Immunolocalization of POMGnT1 in the mouse neural retina. Double immunolabeling of mouse retinal sections for POMGnT1 (**A, B, D, E, G**; red), cone arrestin (cArr; **D, I**; green), or the specific Golgi marker, GM130 (**F, G,** green; **I,** red) was performed. POMGnT1 appeared immunoreactive in the IS layer of photoreceptors (**A**, **E**; asterisk). Nuclei stained with 4',6-diamidino-2-phenylindole (DAPI) are displayed in blue (**B–D, I**). **C**: A negative control without a primary antibody did not show any signal in the IS layer. **D**: POMGnT1 was localized in the myoid portion of cones, where its immunostaining superimposes on cArr labeling (arrow). A portion of the IS layer from **G** is magnified in **H**, where POMGnT1 is shown to colocalize with GM130 within the myoid portion (yellow), where labeling of the latter is superimposed on cArr immunoreactivity (**I**; arrow). POMGnT1 red puncta in the IS in **D** and **I** not overlapped with cArr are presumed to belong to rod photoreceptors. Arrowheads point to non-specific immunostaining of retinal vessels with secondary antibodies to mouse immunoglobulin G in **A**, **C**, **E**. IS = inner segments; ONL = outer nuclear layer; OPL = outer plexiform layer; INL = inner nuclear layer; IPL = inner plexiform layer; GCL = ganglion cell layer; OS = outer segments, m = myoid; n = nucleus. Each bar equals 20 µm, except in **D**, **H**, and **I**: 5 µm.

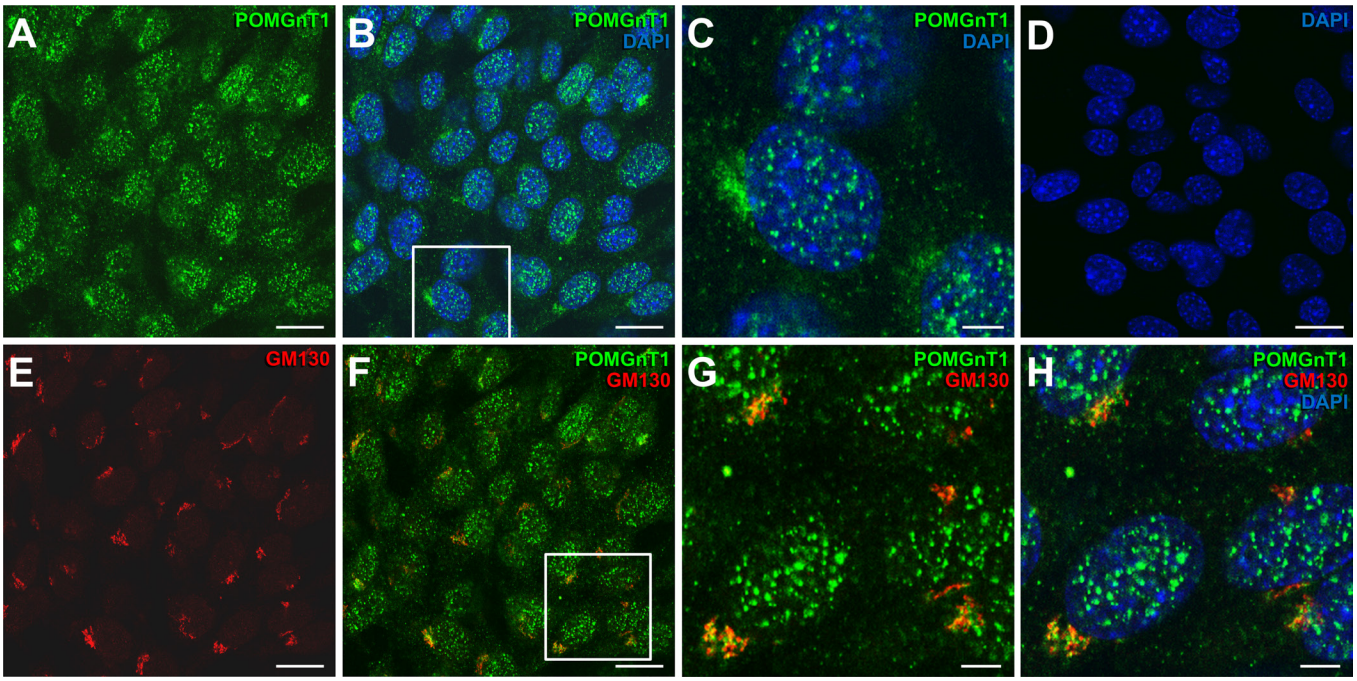

Figure 3. Immunolocalization of POMGnT1 in the 661W photoreceptors. Cells were doubly immunolabeled with antibodies specific to POMGnT1 (**A**, green) and to the Golgi marker GM130 (**E,** red). 4',6-Diamidino-2-phenylindole (DAPI)-stained nuclei are shown in blue (**B, C, D, H**). **D**: A negative control without a primary antibody to POMGnT1 did not show any signal. **F**: POMGnT1 immunoreactivity was distributed between the nucleus and the cytoplasm in 661W cells and colocalized with GM130 in the cytoplasm (yellow). Magnified views in **C, G** and **H** correspond to boxed areas in **B** and **F**. Each bar equals 20 µm, except in **C, G**, and **H**: 5 µm.

was found to distribute in dense, highly immunoreactive elements clustered at one pole of each nucleus stained with DAPI (Figure 3C,H), a location that was compatible with that of the Golgi complex. To investigate this possibility, we performed double immunolabeling for POMGnT1 (Figure 3A) and the Golgi-resident protein, GM130 (Figure 3E). It was found that in the cytoplasm POMGnT1 was located in the Golgi complex of cultured photoreceptors, given the significant colocalization found between the immunolabeling for these two proteins in this organelle (Figure 3F-H, yellow; PC = 0.70±0.06).

The concentration of the POMGnT1 enzyme found in the nucleus of 661W cells was somewhat surprising. To investigate the possible role of this protein in the nuclear compartment, we assessed whether, in addition to POMGnT1, other proteins of the α-DG O-glycosylation pathway were also located in the nucleus of these cells. We performed double immunolabeling using antibodies to POMGnT1 in combination with specific antibodies against proteins POMT1 (Figure 4A) and POMT2 (Figure 4B). We observed that POMT1 and POMT2, similar to POMGnT1, showed a pattern of cytoplasmic plus nuclear distribution, although both were predominantly nuclear (Figure 4A,B). Although the two proteins were dispersed throughout the cytoplasm, they were found to highly colocalize inside the nucleus (Figure 4C, yellow; PC = 0.88±0.03;). Double immunostaining for POMGnT1 and POMT1 revealed, however, that these two enzymes also colocalized inside the nucleus (Figure 4D,E; PC = 0.71±0.04). In the same fashion, significant colocalization between POMGnT1 and POMT2 was observed upon double immunolabeling of 661W cells for these two enzymes (Figure 4G,H; PC = 0.74±0.04). The POMGnT1 colocalization with POMT1 or POMT2 in the nucleus, however, was less than that of POMT1 with POMT2 (compare PC values). In contrast, no colocalization was essentially found between any of these three glycosyltransferases and the DAPI-stained regions (Figure 3B,C, Figure 4F,I; PC = 0.35±0.04, 0.46±0.03, and 0.46±0.03 for POMGnT1, POMT1, and POMT2, respectively), the latter mainly corresponding to heterochromatin clusters inside the nuclei of the 661W photoreceptors.

Finally, to further analyze the intranuclear location in 661W cells of the three enzymes, we performed a series of immunofluorescence microscopy studies in which antibodies against POMGnT1 (Figure 5A), POMT1 (Figure 5D), and POMT2 (Figure 5G) were used separately in combination with specific antibodies to the euchromatin marker histone

H3 trimethylated on Lys-4 (H3K4me3). This analysis revealed that whereas POMGnT1 did not essentially colocalize with the euchromatin marker (Figure 5B,C; PC = 0.46±0.04), POMT1 was found to be located in the euchromatic regions of the 661W cell nucleus (Figure 5E,F, yellow; PC = 0.88±0.02). In the same fashion, POMT2 significantly immunolocalized to euchromatin in the 661W cells (Figure 5H,I, yellow; PC = 0.76±0.04). However, the essential absence of POMGnT1, POMT1, and POMT2 from heterochromatic areas, as determined by their lack of colocalization with DAPI-stained intranuclear clusters, was corroborated for POMGnT1 and POMT1 by their failure to colocalize with the heterochromatin marker, HP1α (data not shown).

DISCUSSION

Dystroglycanopathies are a group of inherited neuromuscular dystrophies that affect the muscle, CNS, and eye, whose underlying basis is a deficient glycosylation of α-DG [3,4,6-9]. The first gene identified as related to this type of diseases was *POMGNT1*, as causative of MEB [14-17], and later on also of WWS and other, less severe dystroglycanopathies [15,16,18,19,21,41]. Mutations in the gene encoding the POMT1 enzyme were shortly thereafter found to be associated mainly with WWS [42], followed by *POMT2* [43]. In patients with MEB, the molecular weight of α-DG decreases by approximately 60 kDa in skeletal muscle and the brain, which is attributable to α-DG hypoglycosylation derived from loss of function of POMGnT1 [44,45]. Patients with MEB display a variety of ocular abnormalities, including electroretinographic (ERG) changes, retinal dysplasia, myopia, glaucoma, microphthalmia, cataracts, retinal degeneration, detachment, and abnormal vascularization, among many others (OMIM 253280) [46-49]. To date, two *Pomgnt1* knockout (KO) gene mouse models have been generated, the first by retroviral insertion of a gene-trapping cassette into the gene's 5′ untranslated region (UTR) [28], and the second by targeted replacement of a gene fragment containing exon 18 by a *neo* cassette [29]. Both mice models were found to

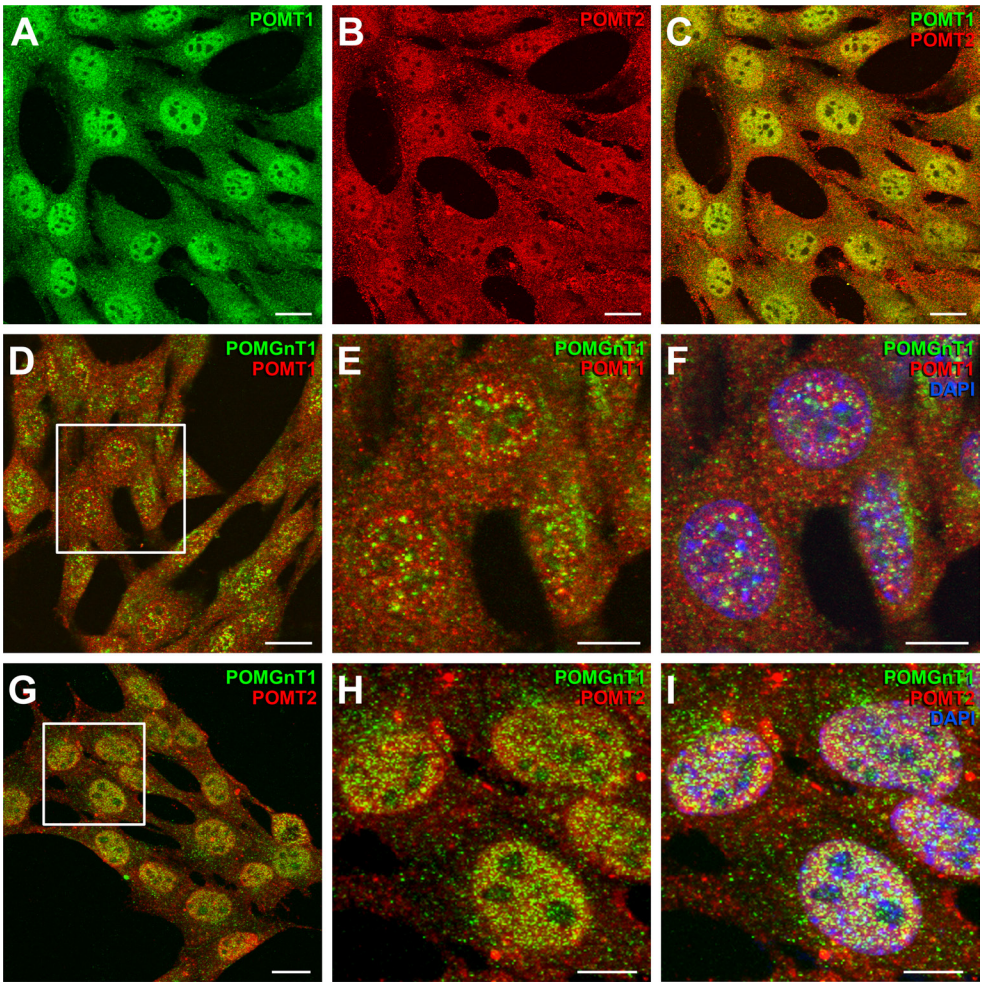

Figure 4. Immunolocalization of POMGnT1 and protein O-mannosyltransferases POMT1 and POMT2 in 661W photoreceptors. Cells were immunostained with antibodies specific for POMT1 (**A**, green) and POMT2 (**B**, red). These two proteins colocalized nearly completely in the nuclei of the 661W cells (**C**, yellow). Double immunostaining with antibodies specific for POMGnT1 (green) and POMT1 (red) is shown in **D** and for POMGnT1 (green) and POMT2 (red) in **G**. Magnified views in **E** and **H** correspond to boxed areas in **D** and **G**, respectively, additionally stained with 4',6-diamidino-2-phenylindole (DAPI) in blue in **F** and **I**. Significant colocalization between POMGnT1 and POMT1 (**E, F**) and between POMGnT1 and POMT2 (**H, I**) is observed. Each bar equals 20 µm, except in **E, F, H**, and **I:** 10 µm.

develop multiple defects in muscle, brain, and eye, mimicking those exhibited by people affected by dystroglycanopathies [28-30,50,51]. Ocular alterations observed in patients with MEB or WWS and in these mouse models underline the relevance of analyzing the distribution of POMGnT1 in the mammalian retina in general, and in photoreceptors in particular, to obtain useful information about POMGnT1 function in retinal cells.

In this work, we detected expression of the *POMGNT1* gene at the mRNA and protein levels in all mammalian species studied, including rodents (mouse and rat), bovine, and primates (monkey and humans). The gene's encoded mRNA transcript and POMGnT1 protein product were also detected in an established photoreceptor cell line, 661W, a mouse retinal transformed cell line with cone properties. In all cases, POMGnT1 showed a molecular mass on immunoblots in agreement with that predicted from its amino acid sequence (75 kDa) and with previous studies. In this context, POMGnT1 has been previously detected with immunoblotting only in the muscle of patients with MEB and healthy individuals, where the protein's molecular mass remained unchanged [52]. Other than this, human POMGnT1 has been detected only as exogenously overexpressed, with immunoblotting in transfected COS-7 cells [21] and with immunocytochemistry in transfected COS-7 and C2C12 cell lines [25,26]. Therefore, our work represents the first instance in which endogenous expression of POMGnT1 is reported in a nervous tissue, the retina, and in a non-transfected cell line, 661W.

The glycosylation of α-DG is a post-translational modification that takes place in the endoplasmic reticulum and the Golgi apparatus. Therefore, as POMGnT1 is involved in the biosynthetic (branched) pathway of two of the three known α-DG O-mannosyl glycan core structures, M1 and M2 (but not M3) [8,9,11], the immunolocalization of this protein in the Golgi apparatus in retinal cells is in agreement with the protein's proposed function in mammalian tissues. The presence of this protein in the Golgi complex

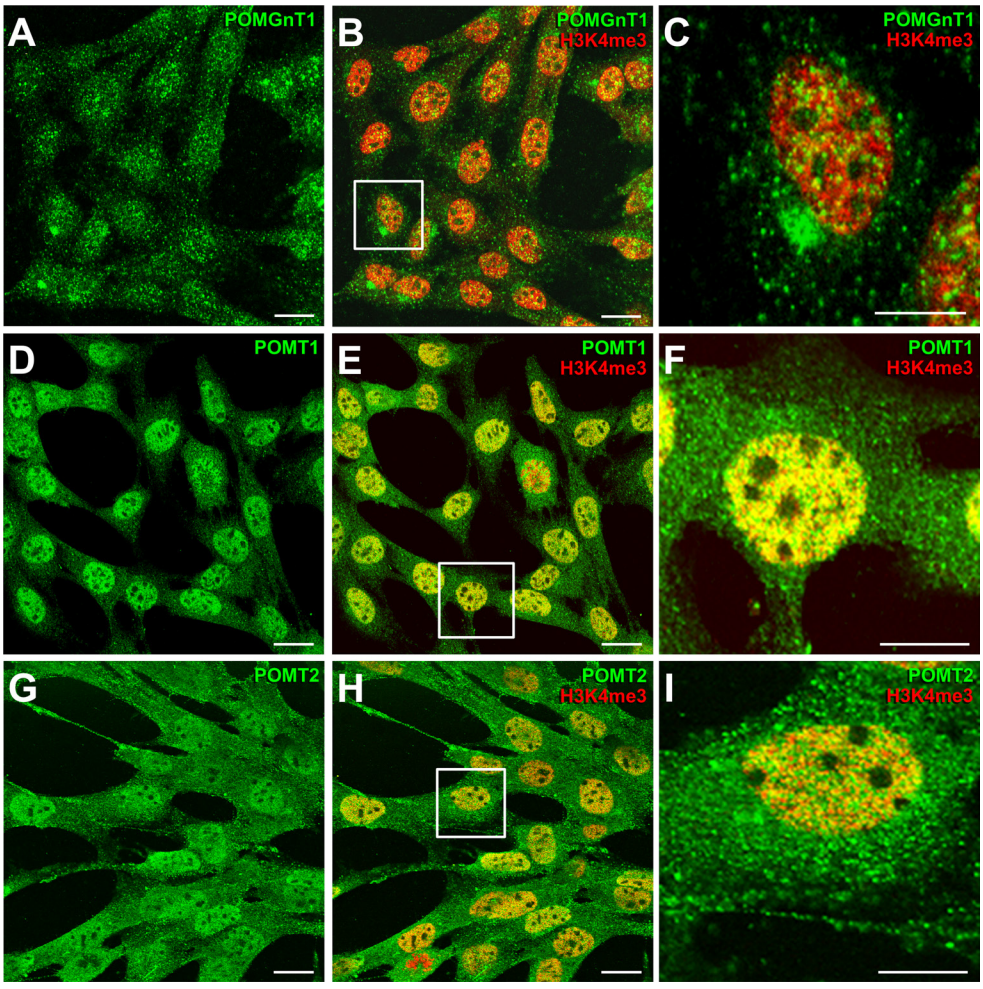

Figure 5. Intranuclear immunolocalization of POMGnT1 together with POMT1 and POMT2 in euchromatin regions of the 661W photoreceptors. Cells were doubly immunostained for POMGnT1 (**A**, green), POMT1 (**D**, green), or POMT2 (**G**, green) and the euchromatin marker H3K4me3 (**B**, **E**, **H**; red). Essentially, no colocalization was found between POMGnT1 and euchromatin (**B**, **C**). In contrast, extensive colocalization was found between POMT1 and euchromatin (**E**, **F**; yellow), and between POMT2 and euchromatin (**H, I**; yellow). Enlarged micrographs in **C**, **F** and **I** correspond to the boxed areas in panels **B**, **E** and **H**, respectively. Each bar equals 20 µm, except in **C**, **F**, and **I**: 10 µm.

had also been observed when exogenously expressed from the human *POMGNT1* gene in transfected C2C12 and COS-7 cells, where POMGnT1 has been shown to colocalize in the Golgi complex and interact with fukutin [25,26], the product of the *FKTN* gene (Gene ID 2218, OMIM 607440) associated with Fukuyama congenital muscle dystrophy, and other dystroglycanopathies. Recently, by cotransfection of the *POMGNT1* gene with that encoding human GOLPH3, it has been demonstrated that the latter mediates the Golgi localization of POMGnT1 in HeLa cells by physically interacting with its cytoplasmic tail, thus targeting and maintaining the enzyme in this organelle [27]. Here, we have shown for the first time the presence of POMGnT1 in the Golgi complex in an adult mammalian tissue, namely, the retina. Specifically, we located POMGnT1 in the myoid region of the inner segments of photoreceptors, the place where the protein biosynthetic machinery is concentrated in this cell type, including ribosomes, the endoplasmic reticulum and the Golgi complex [53]. The location of POMGnT1 in the Golgi complex was conserved in the transformed photoreceptor cell line, 661W. These results support that the function of POMGnT1 in retinal photoreceptors, as established in other cell types and tissues, is to catalyze the second step in the O-mannosyl glycosylation of α-DG, that is, the addition of N-acetylglucosamine units onto O-mannose, previously added by virtue of POMT1 and POMT2 action in the endoplasmic reticulum. It cannot be ruled out at present that other O-mannosylated proteins could serve as well as POMGnT1 substrates remaining to be identified in this tissue.

In the mammalian retina, α-DG has been localized in the outer plexiform layer (OPL), the endothelium of blood vessels, and the inner limiting membrane (ILM) [50,54-56]. In the retina of *Pomgnt1* KO mice, thinning of the outer and inner nuclear layers (ONL and INL, respectively) has been described that was accompanied by absent or residual α-DG glycosylation and pikachurin immunoreactivity in the OPL [50,57,58]. At the physiologic level, a reduction in the amplitude and a delay in implicit time has been found for the ERG a- and b-waves [28,50]. In addition, a disruption of the ILM has been reported together with increased reactivity of astrocytes and Müller glia [30,50]. Our results are thus supportive that POMGnT1 performs a fundamental role in photoreceptors, that is, the glycosylation of α-DG at the Golgi apparatus, which is required for its interaction with its ligand at the OPL extracellular matrix, pikachurin [57,58]. This binding, in turn, has proven to be crucial for the structure and function of synapses established between photoreceptors and their postsynaptic cells [13,59]. The phenotypic and physiologic abnormalities observed in *Pomgnt1* KO mice thus indicate a significant dysfunction originating from hypoglycosylation of α-DG in photoreceptors and affecting other layers of the retina [28,50].

To date, POMGnT1 has not been detected in the nucleus of any vertebrate tissue or cell line. However, our results obtained with immunoblotting have shown the presence of this protein in the nuclear fraction of the 661W photoreceptors, and immunocytochemical studies have revealed a concentration of this protein in the nuclear compartment. The formal possibility exists that POMGnT1 is (mis)localized to this organelle due to their lack of normal cell polarity, this derived from their absence of interaction with RPE cells. This has been used to explain the distribution of cone opsins all over the cell, including the nucleus, in 661W photoreceptors given their lack of outer segments [34]. Although we cannot rule out this possibility, POMGnT1 is not the first instance of a protein involved in the glycosylation of α-DG being detected inside the nucleus, since fukutin has been localized to this compartment in addition to the endoplasmic reticulum and the Golgi complex in two human cancer cell lines, HeLa (cervical cancer) and ZR-75-1 (mammary tumor) [60]. As in these cell lines α-DG is not detected in the nucleus, a new function has been proposed for fukutin in this compartment other than involvement in glycosylation of α-DG, such as the suppression of cell proliferation [60]. In contrast, β-DG, but not α-DG, has been detected in the nucleus of breast [61] and prostate cancer (tissue and cell lines) [62,63], as well as in several well-known, established cell lines of many different tissue and organ origins, such as C2C12 mouse myoblasts [64], PC12 rat pheochromocytoma [65], A549 (lung cancer), Hepa-1 (mouse liver), and even HeLa cells [66]. In several of these cell lines, (unglycosylated) β-DG has been found to form part, as an inner nuclear membrane protein, of a nuclear complex with dystrophin (Dp71 isoform), α-sarcoglycan, α-dystrobrevin, and α-syntrophin, devoid of α-DG and whose proposed function would be to act as a nuclear scaffolding complex [67-69].

We have made our best attempt to detect in the nuclei of 661W photoreceptors β-DG, as well as α-DG, either glycosylated or unglycosylated (core α-DG), with negative results (data not shown). However, we observed β-DG and glycosylated α-DG in the cytoplasm of these cells, by using conventional antibodies widely used by many authors (data not shown). We then tried to localize in 661W cells other proteins involved in α-DG glycosylation, such as POMT1 and POMT2, which is responsible for catalyzing the preceding step of this pathway [22,23]. These two proteins were found, in addition to the cytoplasm (where they would carry out α-DG O-mannosylation) in the nuclei of 661W cells, where they colocalized with the POMGnT1 enzyme. Interestingly,

POMT1 and POMT2 colocalized nearly completely with each other, which speaks in favor of these two proteins forming a heterodimer required for their protein O-mannosyltransferase activity [22,23] and highly colocalized with the euchromatic—but not heterochromatic—regions within the nucleus of 661W cells. To our knowledge, this is the first instance of dystroglycanopathy-related proteins being associated with chromatin and perhaps exerting an indirect transcriptional regulatory role, distinct from their function in α-DG glycosylation in the cytoplasm.

As a difference compared to photoreceptors in their natural context of the retina, 661W is a cell line derived from a retinal tumor, which have undergone transformation as a consequence of expressing the SV40 T viral oncoprotein, and thus exhibit an unlimited capacity of cell division [33]. An increasing number of studies have unveiled that many tumor tissues and cell lines, including prostate, digestive system, renal cancer, and glioblastomas, exhibit a downregulation of α-DG and β-DG protein levels [70-73] or a hypoglycosylation of α-DG[73-78]. Recently, POMGnT1 levels have been found altered in human glioblastomas in a correlated fashion with their tumor grade (malignancy) [79]. This ample set of results strongly point to a role of DG levels of expression or glycosylation (or both) in modulation of cellular proliferation. In this context, a picture has emerged in recent years in which tumor progression involves downregulation of dystroglycanopathy-related genes, this contributing to their detachment from the extracellular matrix and acquisition of migratory properties. This is the case of *LARGE* (Gene ID 9215, OMIM 603590); *LARGE2* (Gene ID 120071, OMIM 609709); *B3GNT1* (Gene ID 11041, OMIM 605517), and *ISPD* (Gene ID 729920, OMIM 614631) in prostate cancer [73,75,80], tongue cancer [74], or renal carcinoma [76].

Given that DG appears to be absent from the nucleus of 661W cells, the possibility can be raised that POMGnT1 could be involved, possibly in cooperation with the POMT1/2 heterodimer, in the glycosylation of yet-to-be identified nuclear proteins perhaps with a modulatory role of cell adhesion/migration or proliferation abilities. Recently, a new set of proteins have been found to be O-mannosyl glycosylated in the mouse brain in a POMGnT1-dependent fashion [81]. These proteins encompass mainly cadherins and plexins, although not any known transcription factor. However, a protein-tyrosine-phosphatase named RPTPζ/phosphacan has recently emerged as an apparent new substrate for POMGnT1 [82]. It is therefore tempting to speculate that POMGnT1 could indirectly influence these cellular properties by modulating intranuclear signal transduction. Further research is needed to clarify the role of POMGnT1 in the nucleus of dividing photoreceptors, where this protein is not found in its natural retinal environment.

APPENDIX 1.

STR analysis. To access these data, click or select the words "Appendix 1".

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